REMARKS

Claims 1–34 are cancelled and Claims 35–53 are pending.

Claims 37-38, 40, 42, 44, 46, 48, 50 and 52-53 are allowed.

Claims 35, 39, 41, 43, 45, 47, 49 and 51 are rejected.

Claim 35 is amended to delete reference to the sequence SLILLGV (amino acids 107-113, SEQ ID NO: 2).

Applicant believes that no new matter is added by way of amendment.

I. Allowance of Claims 37-38, 40, 42, 44, 46, 48, 50 and 52-53

Applicant notes with appreciation the allowance of Claims 37-38, 40, 42, 44, 46, 48, 50 and 52-53.

II. Rejection of Claims 35, 39, 41, 43 and 45 under 35 U.S.C. §102(e)

Claims 35, 39, 41, 43 and 45 stand rejected under 35 U.S.C. §102(e) as being anticipated by U.S. Pat. No. 6,117,654 to Yang Pan, as evidenced by Bost et al. (1988). The Examiner argues, in essence, that the presence of the sequence "ILLGV" in the TANGO-77 protein of the '654 patent, which patent also discloses antibodies to TANGO-77, means that the '654 patent inherently discloses antibodies that would cross-react with IL-1ζ of the present application at an epitope comprising the sequence "ILLGV", thus anticipating the rejected claims.

Although Applicant respectfully disagrees with the rejection, as detailed below, the claims are nonetheless amended herein to eliminate the rejected subject matter.

Claim 35, as presented in the response dated August 1, 2006, encompassed a binding compound that specifically binds to a polypeptide consisting of the amino acid sequence of IL-1ζ (SEQ ID NO: 2) at an epitope comprising, *inter alia*, the sequence SLILLGV (amino acids 107-113 of SEQ ID NO: 2). Claim 35 is not directed to all antibodies that bind to the recited epitope sequences, but instead covers antibodies that specifically bind to the epitopes within the context of the sequence of IL-1ζ.

An antibody that binds to the epitope ILLGV in the context of TANGO-77, as postulated in the rejection, would not necessarily bind to that same sequence in the context of IL-1ζ. Specifically, an antibody that binds to ILLGV within SPILLVG (TANGO-

77) would not necessarily bind to ILLGV within SLILLVG (IL-1ζ). The amino acid proline (P), for example, is known in the art to disrupt protein structures, such as alpha-helices. *See* Vanhoof *et al.* (1995) *FASEB J.* 9:736 (attached as Exhibit 1). This could result in a significant structural difference between TANGO-77 and IL-1ζ in the immediate vicinity of the proposed ILLGV epitope.

Although it is *possible* that an antibody to TANGO-77 at ILLGV might cross-react to IL-1ζ, this isn't certain. The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. MPEP §2112, citing *In re Rijckaert*, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). The fact that Bost et al. (1998) discloses one example of a cross-reactive antibody does not support the general argument that such cross-reactive antibodies must always exist for any given 4-6 amino acid region of sequence identity.[1]

Despite the foregoing argument, and solely to facilitate prosecution, Claim 35 is amended herein to eliminate the rejected subject matter. Applicant reserves the right to pursue this subject matter in a subsequent, related application.

In light of this amendment to Claim 35, and thus to dependent claims 39, 41, 43 and 45, Applicant submits that the rejection under 35 U.S.C. §102(e) is overcome. Because Claim 36 was objected to for being dependent from rejected Claim 35, Applicant further requests withdrawal of this objection in light of the amendment to Claim 35.

III. Rejection of Claims 47, 49 and 51 under 35 U.S.C. §103

Claims 47, 49 and 51 stand rejected under 35 U.S.C. §103 as being obvious in light of U.S. Pat. No. 6,117,654 to Yang Pan, as evidenced by Bost et al. (1988), in view of the Stratagene catalog (1988, page 39).

Because the obviousness rejection is based in large part on the '654 patent, and the amendment to Claim 35 eliminates the subject matter allegedly anticipated by the

[1] The Abstract of Bost et al. states that based on a previously identified 6-amino acid sequence homology between IL-2 and HIV envelope protein, "it is conceivable that" there may be antibodies that cross-react between the two proteins. It is only the experimental work disclosed in that publication that establishes the fact of cross-reactive antibodies in that particular situation.

'654 (as discussed *supra*), Applicant respectfully requests that the rejection under 35 U.S.C. §103 be withdrawn.

Conclusion

Applicant's current response is believed to be a complete reply to all the outstanding issues of the latest Office action. Further, the present response is a bona fide effort to place the application in condition for allowance or in better form for appeal. Accordingly, Applicant respectfully requests reconsideration and passage of the amended claims to allowance at the earliest possible convenience.

Applicant believes that no additional fees are due with this communication. Should this not be the case, the Commissioner is hereby authorized to debit any charges or refund any overpayments to DNAX Deposit Account No. 04-1239.

If the Examiner believes that a telephonic conference would aid the prosecution of this case in any way, please call the undersigned.

Respectfully submitted,

Date: 8 March 2007

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Enclosure: Exhibit 1 (Vanhoof *et al.* (1995) *FASEB J.* 9:736)

Proline motifs in peptides and their biological processing

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ABSTRACT **Many biologically important peptide sequences contain proline. It confers unique conformational constraints on the peptide chain in that the side-chain is cyclized back onto the backbone amide position. Inside an α-helix the possibility of making hydrogen bonds to the preceding turn is lost and a kink will be introduced. The conformational restrictions imposed by proline motifs in a peptide chain appear to imply important structural or biological functions as can be deduced from their often remarkably high degree of conservation as found in many proteins and peptides, especially cytokines, growth factors, G-protein-coupled receptors, V3 loops of the HIV envelope glycoprotein gp120, and neuro- and vasoactive peptides. Only a limited number of peptidases are known to be able to hydrolyze proline adjacent bonds. Their activity is influenced by the isomeric state (cis-trans) as well as the position of proline in the peptide chain. The three proline specific metallo-peptidases (aminopeptidase P, carboxypeptidase P and prolidase) are activated by Mn^{2+}, whereas the three serine type peptidases cleaving a post proline bond (prolyl oligopeptidase, dipeptidyl peptidase IV, and prolylcarboxypeptidase) share the sequential order of the catalytic Ser-Asp-His triade, which differentiates them from the chymotrypsin (His-Asp-Ser) and subtilisin (Asp-His-Ser) families. An endo or C terminal Pro-Pro bond and an endo pre-Pro peptide bond possess a high degree of resistance to any mammalian proteolytic enzyme.—Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., Scharpé, S. Proline motifs in peptides and their biological processing. *FASEB J.* 9, 736–744 (1995)**

Key words: cytokines · dipeptidyl peptidase IV · prolyl oligopeptidase · cis-trans isomerization · substance P · IgA proteases

AMONG THE 20 AMINO ACIDS that are coded in protein synthesis, proline occupies a unique position. The only mammalian imino acid imposes strong restraints on the conformation of a peptide chain, since, unlike the other amino acids, the α-nitrogen atom of proline is part of the rigid pyrrolidine ring and, at the same time, is covalently bound by means of a secondary amide bond to the preceding amino acid (1). When present inside an α-helix, proline also sterically prevents the amide nitrogen of its C-terminal neighbor from making a hydrogen bond with a carbonyl in the preceding turn of the helix and thus introduces a kink of 20° or more in the α-helix (2) (**Fig. 1**). Moreover, unlike other amino acids, proline and hydroxyproline can more readily introduce structural heterogeneity since the X-proline (X being any amino acid) or X-hydroxyproline bond can adapt either the stereoisomeric cis or trans conformation (3).

PROLINE AND PEPTIDE CONFORMATION

Proline is believed to be important in one of the earliest events in protein biochemistry, the process of protein folding, which is necessary to achieve the physiologically active three-dimensional structure. By studying the folding process of denatured proteins, it has been found that several kinetic phases can be distinguished in this process (4). *cis-trans* isomerization of X-Pro bonds turned out to be the rate-limiting slow phase of protein renaturation (**Fig. 2**) (5).

Two sequence-unrelated families of proteins, the cyclophilins (cyclosporin A binding proteins) and the FKBP (FK506/rapamycin-binding proteins), possess peptidyl-prolyl *cis-trans* isomerase (EC 5.2.1.8) activities. Some of them proved to be part of a network of foldases that regulate folding, assembly, and trafficking of proteins in the cellular environment (6). In relation with these findings, it has been suggested that the peptidyl-prolyl *cis-trans* isomerases are relevant to the regulation of intracellular signaling events in T cell activation.

The exceptional properties of proline render its presence inside α-helices energetically unfavorable. Nevertheless, proline has been found to occur with relatively high frequency in the putatively α-helical transmembrane segments of many integral membrane proteins that function as receptor subunits or transporters (7).

The entire superfamily of G-protein-coupled receptors contains a series of membrane-embedded and highly con-

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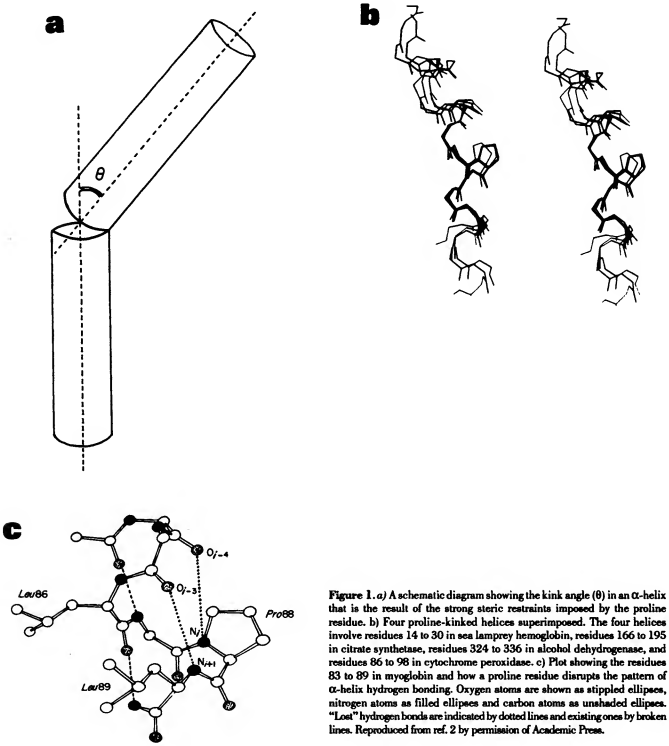

Figure 1. *a)* A schematic diagram showing the kink angle (θ) in an α-helix that is the result of the strong steric restraints imposed by the proline residue. b) Four proline-kinked helices superimposed. The four helices involve residues 14 to 30 in sea lamprey hemoglobin, residues 166 to 195 in citrate synthetase, residues 324 to 336 in alcohol dehydrogenase, and residues 86 to 98 in cytochrome peroxidase. c) Plot showing the residues 83 to 89 in myoglobin and how a proline residue disrupts the pattern of α-helix hydrogen bonding. Oxygen atoms are shown as stippled ellipses, nitrogen atoms as filled ellipses and carbon atoms as unshaded ellipses. "Lost" hydrogen bonds are indicated by dotted lines and existing ones by broken lines. Reproduced from ref. 2 by permission of Academic Press.

served proline residues, which are likely to introduce kinks into the transmembrane helices (8). Studies using site-directed mutagenesis demonstrate that these residues play key roles in receptor expression, ligand binding, and receptor activation (9). These findings led to the proposal that *cis-trans* isomerization of the X-Pro bond could provide the conformational change in protein structure necessary for the regulation (opening/closing) of a transport channel. In addition, intrahelical prolines provide liganding sites for cations via exposure of the backbone carbonyl oxygen atoms of residues i-3 and i-4 (relative to the proline) (10). Thus, the proline-containing helix may play a role in proton pumps, sodium/potassium pumps, calcium pumps, and calcium regulatory sites. This hypothesis is strongly supported by the site-directed Pro-mutant of the Ca^{2+}-ATPase where the Ca^{2+}-uptake was strongly affected by the mutation (11).

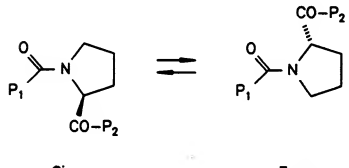

Figure 2. *cis* (left) and *trans*-conformation (right) of the X-Pro peptide bond with a rotation of 180° around the C-N imide bond. The reaction is catalyzed by peptidyl-prolyl *cis-trans* isomerase (6).

PROLINE IN PEPTIDES INTERFERING IN IMMUNOMODULATION AND COAGULATION

The activity of the immune system can be modulated by a variety of natural and synthetic peptides. It is striking that many of these small peptides carry proline residues in their sequence (**Table 1**). Although the action mechanism of immunostimulating or immunosuppressive peptides is largely unknown, they have valuable clinical potency. Exploration of the importance of the proline signal for the biological activities of these peptides may lead to a broader understanding of the immune response and to the development of new potent and biocompatible immunomodulators.

The bactenecins are unusual peptides with regard to sequence and biological function. These highly cationic peptides of the large granules of neutrophils are characterized by an abundance of prolyl and arginyl residues, arranged as repeating Arg-Pro-Pro motifs alternating with apolar residues in bactenecin 5, and by Pro-Arg-Pro triplets spaced by a single hydrophobic residue in bactenecin 7 (21). The bactenecins' capacity to permeabilize membranes suggests an important role in antimicrobial host defense and in autoimmunity (22).

The same Pro-Arg-Pro sequence is found at the N-terminus of the fibrin α chain. The tetrapeptide Gly-Pro-Arg-Pro can prevent fibrin from polymerizing and associating with platelets during thrombin-induced release of platelet granule contents, which suggests a functional role of the fibrin α chain N-terminus in fibrin polymerization (13, 14, 18). Furthermore, the same terminus serves as a recognition site for the integrin CD11c/CD18 on stimulated neutrophils (23).

THE -X-Pro- AND -X-Pro-Y- MOTIFS AS TARGETS FOR PATHOGENIC BACTERIA AND VIRUSES

Although repetitive proline motifs inside peptide chains often play a structural role, as illustrated by the collagen family, they may also serve as a protective and binding element (reviewed in ref. 24). Arranged as multiple proline-rich repeats, they encourage an extended conformation resembling the polyproline II helix, which consists of three proline residues per turn (25). Proline-rich tandem repeats constitute almost the entire sequence of the proline-rich-proteins, a major constituent of human saliva biologically functioning as multivalent binder (26) and thought to be responsible for the neutralization of harmful effects of tannins present in foods (e.g., fruit, berries, cocoa, sorghum) and beverages (e.g., coffee, tea, beer, wine, cider). This binding ability is understood to derive from the restricted mobility of proline, which reduces the unfavorable entropy loss of peptides on binding, the flat hydrophobic surface of proline, and the unique characteristics of the amide bond preceding proline (24).

Proline residues inside a peptide chain may also act as a mode of protection against nonspecific proteolytic degradation. It is known that IgA mediates the immunity to infec-

TABLE 1. *Proline in peptides with immunopharmacological activities*[a]

Structure	Activity
Thr-Lys-**Pro**-Arg (tuftsin)	Enhances the phagocytosis by monocytes and macrophages (12)
Gly-**Pro**-Arg-**Pro**	Prevents polymerization of fibrin and association with platelets (13, 14)
Leu-**Pro**-**Pro**-Ser-Arg	Drives B lymphocytes to immunoglobulin secretion (15)
AcSer-Asp-Lys-**Pro**	Controls proliferation of the hematopoietic stem cell (16)
Asp-Ser-Asp-**Pro**-Arg	Tonical use in the treatment of ocular and nasal allergies (17)
His-**Pro**-**Pro**-Gly-Phe	Delays the growth of Lewis carcinoma in mice and inhibits the formation of lung metastases (18)
Val-Glu-**Pro**-Ile-**Pro**-Tyr	In vitro immunostimulating activity on murine macrophages and human monocytes (19)
Tyr-Val-**Pro**-Gly-Phe-**Pro**	Strong immunosuppressor activity (17)
Val-Glu-Ser-Tyr-Val-**Pro**-Leu-Phe-**Pro**	Modulates the antibody responses of mice and affects the maturation of T cell precursors (19)
Gly-**Pro**-Thr-Gly-Thr-Gly-Ser-Lys-Cys-**Pro**	Immuno-enhancing activity (20)
Arg-Phe-Arg-**Pro**-**Pro**-Ile-Arg-Arg-**Pro**-**Pro**-Ile-Arg-**Pro**-**Pro**	Bactenecin 5 (1–14), potent antimicrobial and cytotoxic activity (21, 22)
Arg-Arg-Ile-Arg-**Pro**-Arg-**Pro**-**Pro**-Arg-Leu-**Pro**-Arg-**Pro**-Arg	Bactenecin 7 (1–14), potent antimicrobial and cytotoxic activity (21, 22)

[a]Numbers in parentheses refer to References.

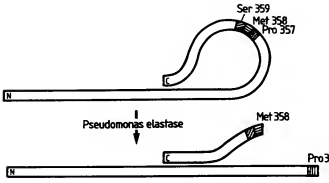

Figure 3. Schematic representation of α_1 antitrypsin inhibitor as a trap where the Met^{358}-Ser^{359} bond functions as a bait for serine proteases such as human neutrophil elastase. *Pseudomonas* elastase irreversibly destroys the inhibitor by hydrolysis of the preceding Pro^{357}-Met^{358} linkage in the strained loop of the molecule.

tion at mucosal surfaces. Important pathogens of mucosal surfaces, such as *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, secrete proteases with a marked specificity for IgA1. They show a remarkable, restricted substrate specificity to cleave the heavy chain of IgA1 in the hinge region at Pro-X positions releasing the intact Fab and Fc fragments. The production of these proline-directed metalloproteases (EC 3.4.24.13) and serine proteases (EC 3.4.21.72) seems to be associated with the virulence of these organisms, since other, phylogenetically related but not pathogenic, bacteria do not produce these IgA proteases (27). Other pathogenic bacteria such as *Gardnerella vaginalis* also produce proline-specific peptidases. Although the target substrates of these peptidases have not yet been described, the measurement of proline aminopeptidase levels in vaginal secretions is used in the diagnosis of bacterial vaginosis (28).

These bacterial IgA proteases are not hindered by proteinase inhibitors such as α1 antitrypsin. This resistance may be the result of the inactivation of this inhibitor, as has been shown for Pseudomonas aeruginosa elastase (EC 3.4.24.26). This metallo-endoprotease can inactive α1 antitrypsin by splitting the peptide bond Pro357-Met358 exposed in the strained loop of α1 antitrypsin (**Fig. 3**), which functions physiologically as a bait for human neutrophil elastase. During pseudomonal disease, the breakdown of the α1 antitrypsin trap permits the endogeneous serine proteases to cause tissue destruction (29).

During the replication of retroviruses, such as human immunodeficiency virus (HIV),[2] *gag* and *gag-pol* gene products are translated as polyproteins (55-kDa precursor and 160-kDa precursor). These polyproteins are subsequently processed by a virally encoded aspartic protease to yield several structural proteins of the viral core together with essential viral enzymes, including the aspartic protease itself (p11). The aspartic protease p11 is probably autocatalytically released from the 160-kDa precursor by hydrolysis of two Phe-Pro bonds (30). This aspartic protease, essential for viral replication, is not only an important drug target for the treatment of HIV infection but also exceptional in that it is able to split internal pre-Pro peptide bonds, such as Phe-Pro and Tyr-Pro. To date no mammalian or bacterial enzyme is known that can hydrolyze this type of linkage.

The V3 loop of the HIV envelope glycoprotein gp120 is critical for the cell entry of the virus. In the crown of this V3 loop, despite its high variability in amino acid sequence, highly conserved Arg-Pro and Gly-Pro motifs have been identified that are strongly conserved in more than 90% of the isolates characterized and remain unchanged in vivo in a given individual over the years of infection (31). The remarkable conservation of these motifs indicates a constant selective pressure to preserve them because of their probably crucial role in HIV tropism.

PROLINE-DIRECTED ARGINYL CLEAVAGE

The conformational constraint induced by proline residues in a peptide usually restricts proteolysis. However, depending on its position in the peptide chain, the presence of proline can make a peptide bond more susceptible to enzymatic hydrolysis. Important physiological examples are found in the coagulation cascade, where the action of thrombin is mediated by the presence of a proline residue just before an arginine residue, resulting in a proline-directed monobasic arginyl cleavage (**Table 2**). This mechanism can be considered as an alternative to a trypsin-like cleavage between two basic residues followed by a removal of C-terminal Lys or Arg by a basic carboxypeptidase (32, 33).

In the reverse situation when Arg precedes Pro, the arginyl linkage is usually resistant to trypsin. However, when this Arg-Pro motif is preceded by Gln the arginyl bond becomes susceptible again to trypsin action (34).

AN AMINOTERMINAL X-Pro SHIELD IN CYTOKINES AND GROWTH FACTORS

Exploration of the amino acid sequences of the proteins registered in international data banks reveals that a remarkable number of cytokines and growth factors share an X-Pro sequence at their aminoterminus (**Table 3**). The Ala-Pro motif is found at the N-termini of interleukin (IL)-1 β, IL-2, granulocyte macrophage colony stimulating factor, and erythropoietin. The N-terminal X-Pro sequence may not only contribute to the biological activity, as demonstrated for IL-1 β (35, 36) and IL-2 (37), but also serve as a structural protection against nonspecific proteolytic degradation analogous to C-terminal amidation, acetylation, or N-terminal cyclization to pyroglutamic acid. The striking degree of conservation seems to reflect an evolutionary pressure toward this X-Pro motif.

[2]Abbreviations: HIV, human immunodeficiency virus; IL, interleukin; ACE, angiotensin-converting enzyme.

Growth hormone and prolactin (38), two evolutionary related proteins, are fortified against proteolytic attack at both ends of the peptide chain. The protection at the aminoterminus is obtained by two highly conserved proline residues at position 2 and 5, respectively. A comparable situation is found in tumor necrosis factor β, but at positions 2 and 7. Conserved aminoterminal proline motifs are also shared by tissue plasminogen activator, precursor proteins such as trypsinogen and procolipase, whereas the α subunit of human chorionic gonadotropin, luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone is found with and without the N-terminal X-Pro motif (39).

Only two proline-specific aminopeptidases, respectively aminopeptidase P and dipeptidyl peptidase IV (**Fig. 4**) have a substrate specificity towards the N-terminal X-Pro motif and may, possibly after a preceding endoproteolysis (40, 41), be important in the modulation of biological activity of some of the cytokines and growth factors given in Table 3.

PROLINE PEPTIDASES: Mn^{2+}-ACTIVATED METALLO-EXOPEPTIDASES AND A NEW FAMILY OF SERINE PROTEASES

Besides the dipeptidases prolidase (EC 3.4.13.9) and prolinase (EC 3.4.13.8), only a few enzymes that are involved in the cleavage of proline-containing peptide bonds have been demonstrated in mammalia (42): five exopeptidases, aminopeptidase P (EC 3.4.11.9), dipeptidyl peptidase II (EC 3.4.14.2) and IV (EC 3.4.14.5), membrane carboxypeptidase P (EC 3.4.17.16), lysosomal prolylcarboxypeptidase (EC 3.4.16.2), and one endopeptidase, prolyl oligopeptidase (EC 3.4.21.26) (Fig. 4). Their properties are recently reviewed by Yaron et al. (43).

No mammalian enzyme has been identified with a specificity restricted to a prolyl aminopeptidase activity, and recent studies indicate that leucyl-aminopeptidase activity and prolyl-aminopeptidase activity originate from the same enzyme (44). Prolinase, too, cannot be considered as a proline-selective enzyme, because, for its activity, the N terminal proline may be substituted by other amino acids. On the other hand, prolidase and aminopeptidase P proved to possess a high specificity for the secondary amide bond in the aminoterminal X-Pro motif, which they cleave only in *trans* conformation. Both enzymes occur in many species and tissues. While prolidase seems to be involved mainly in amino acid recycling, there are several reasons to expect an important physiological role for aminopeptidase P in conjunction with dipeptidyl peptidase IV and prolyl oligopeptidase, as will be discussed below. An activity analogous to that of aminopeptidase P but situated at the C terminus is exerted by membrane carboxypeptidase P and lysosomal prolylcarboxypeptidase (Fig. 4). It is striking that the three mammalian proline metallopeptidases, aminopeptidase P (45, 46) membrane carboxypeptidase P (47), and prolidase are activated by Mn^{2+}.

Comparative-sequence analysis indicates that the three serine-type peptidases involved in the cleaving of proline containing bonds, prolyl oligopeptidase (48), dipeptidyl peptidase IV (49), and prolylcarboxypeptidase (50), while sharing the same sequential ordening of the catalytic triad, exhibit a different sequential ordening to that of the chymotrypsin family (His-Asp-Ser) and the subtilisin family (Asp-His-Ser) (51).

Dipeptidyl peptidase IV removes the N-terminal dipeptides from polypeptides carrying an unsubstituted N-terminus and a penultimate proline residue in *trans* conformation. This enzyme is widely distributed in mammalian tissues and is involved in intestinal assimilation and renal handling of proline containing peptides (43), adhesion phenomena (52), neoplastic cell transformation (53), and several types of immunological reactions. The recognition of dipeptidyl peptidase IV on the cell surface of lymphocytes as the human T lymphocyte activation antigen CD26, its role in T cell activation and proliferation (54–58), its function as binding protein for adenosine deaminase (59, 60), and evidence of its involvement in HIV infections (31, 61, 62) are strong stimuli for further exploration of its biological functions. Dipeptidyl peptidase IV is exceptional, too, in combining a serine-type protease mechanism with an exopeptidase activity, whereas most exopeptidases are metalloproteases.

The only proline endopeptidase known so far in mammalia is prolyl oligopeptidase, which cleaves the peptide bond at the carboxyl side of proline. The substrate specific-

TABLE 2. *Sites of cleavage by thrombin in peptides from the coagulation cascade (human)*

Protein	Subsite	
	P_5 P_4 P_3 P_2 P_1	P_1' P_2' P_3' P_4' P_5'
Fibrinogen (Aα)	-Val-Arg-Gly-**Pro**-Arg^{19}	Val-Val-Glu-Arg-His-
Factor XIII	-Glu-Gly-Val-**Pro**-Arg^{36}	Gly-Val-Asx-Leu-Glx-
Factor VIII	-Ala-Ile-Gly-**Pro**-Arg^{740}	Ser-Phe-Ser-Gln-Asn-
	-Asn-Gln-Ser-**Pro**-Arg^{1689}	Ser-Phe-Gln-Lys-Lys-
Factor V	-Pro-Leu-Ser-**Pro**-Arg^{1018}	Thr-Phe-His-Pro-Leu-
Prothrombin	-Met-Val-Thr-**Pro**-Arg^{155}	Ser-Glu-Gly-Ser-Ser-
	-Phe-Phe-Asn-**Pro**-Arg^{286}	Thr-Phe-Gly-Ser-Gly-
Protein C	-Gln-Val-Asp-**Pro**-Arg^{169}	Leu-Ile-Asp-Gly-Lys-
Procarboxypeptidase U (34)	-Thr-Val-Ser-**Pro**-Arg^{114}	Ala-Ser-Ala-Ser-Tyr-

TABLE 3. *N-terminal amino acid sequences of mature cytokines and growth factors*

	1	2	3	4	5	6	7	8
Interleukin-1β								
human	Ala	Pro	Val	Arg	Ser	Leu	Asn	Cys
bovine	Ala	Pro	Val	Gln	Ser	Ile	Lys	Cys
mouse	Val	Pro	Ile	Arg	Gln	Leu	His	Tyr
Interleukin-2								
human, pig, sheep	Ala	Pro	Thr	Ser	Ser	Ser	Thr	Lys
rat	Ala	Pro	Thr	Ser	Ser	Pro	Ala	Lys
mouse	Ala	Pro	Thr	Ser	Ser	Ser	Thr	Ser
Interleukin-5								
human	Ile	Pro	Thr	Glu	Ile	Pro	Thr	Ser
Interleukin-6								
human	Val	Pro	Pro	Gly	Glu	Asp	Ser	Lys
bovine	Gly	Pro	Leu	Gly	Glu	Asp	Phe	Lys
rat, mouse	Phe	Pro	Thr	Ser	Gln	Val	Arg	Arg
Interleukin-10								
human	Ser	Pro	Gly	Gln	Gly	Thr	Gln	Ser
Interleukin-13								
human	Gly	Pro	Val	Pro	Pro	Ser	Thr	Ala
Granulocyte-macrophage colony-stimulating factor								
human	Ala	Pro	Ala	Arg	Ser	Pro	Ser	Pro
bovine	Ala	Pro	Thr	Arg	Pro	Pro	Asn	Thr
mouse	Ala	Pro	Thr	Arg	Ser	Pro	Ile	Thr
Granulocyte colony-stimulating factor								
human	Thr	Pro	Leu	Gly	Pro	Ala	Ser	Ser
mouse	Val	Pro	Leu	Val	Thr	Val	Ser	Ala
Erythropoietin								
human, mouse	Ala	Pro	Pro	Arg	Leu	Ile	Cys	Asp
Insulin-like growth factor I								
human, bovine, pig, sheep, mouse, chicken	Gly	Pro	Glu	Thr	Leu	Cys	Gly	Ala
Tumor necrosis factor β								
human	Leu	Pro	Gly	Val	Leu	Thr	Pro	Ser
pig	Leu	Pro	Gly	Val	Gly	Leu	Pro	Pro
rabbit	Leu	Pro	Gly	Ala	Glu	Phe	Pro	Pro
Monocyte chemotactic protein I								
human	Glu	Pro	Asp	Ala	Ile	Asn	Ala	Pro
rat	Glu	Pro	Asp	Ala	Val	Asn	Ala	Pro
rabbit	Glu	Pro	Asp	Ala	Val	Asn	Ser	Pro
bovine	Glu	Pro	Asp	Ala	Ile	Asn	Ser	Glu
Granulocyte chemotactic protein II								
human	Gly	Pro	Val	Ser	Ala	Val	Leu	Thr
Growth hormone								
human	Phe	Pro	Thr	Ile	Pro	Leu	Ser	Arg
bovine	Phe	Pro	Ala	Met	Ser	Leu	Ser	Gly
pig, elephant, rat, mouse	Phe	Pro	Ala	Met	Pro	Leu	Ser	Ser
Prolactin								
human	Leu	Pro	Ile	Cys	Pro	Gly	Gly	Ala
bovine	Thr	Pro	Val	Cys	Pro	Asn	Gly	Pro
pig	Leu	Pro	Ile	Cys	Pro	Ser	Gly	Ala
elephant	Ile	Pro	Val	Cys	Pro	Arg	Gly	Ser
rat	Leu	Pro	Val	Cys	Ser	Gly	Gly	Asp
mouse	Leu	Pro	Ile	Cys	Ser	Ala	Gly	Asp
Melanoma growth stimulating activity								
human	Ala	Pro	Leu	Ala	Thr	Glu	Leu	Arg
Interferon inducible protein 10								
human	Val	Pro	Leu	Ser	Arg	Thr	Val	Arg
mouse	Ile	Pro	Leu	Ala	Arg	Thr	Val	Arg

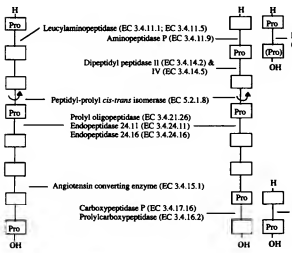

Figure 4. Peptidases and isomerases known in humans to process proline adjacent linkages. Endopeptidase 24.11 needs an hydrophobic residue at the carboxylterminus of proline, while endopeptidase 24.16 prefers Pro-Tyr bonds although Pro is not an absolute requirement.

ity of this serine protease is not totally exclusive, because in some substrates, depending on the adjacent amino acids, two metallopeptidases, neutral endopeptidase 24,11 (EC 3.4.24.11) (63) and neurotensin endopeptidase (EC 3.4.24.16) (64), are also able to cleave some postproline peptide bonds. In addition, prolyl oligopeptidase can process some peptide hormones like angiotensin II and luteinizing hormone-releasing hormone, functioning in these situations as a prolylcarboxypeptidase, although with a much lower catalytic efficiency.

However, not only the presence and position in the peptide chain of an X-Pro linkage but also the isomeric state of this bond must be considered. The different proline-specific peptidases mentioned above will cleave only when the peptide bond preceding the proline residue is in its *trans* conformation (43). This means that the control of the processing of their substrates may in some instances involve a prior conversion of a *cis* conformation to the *trans* position by a peptidyl-prolyl *cis-trans* isomerase (EC 5.2.1.8) (3). The finding that the binding of these proline *cis-trans* isomerases with the immunosuppressants cyclosporin and FK506 is accompanied by a potent inhibition of their isomerase activity (6) may also open new perspectives in the exploration of proline-conditioned peptide hydrolysis.

PROLINE MOTIFS AND CONDITIONED PEPTIDE HYDROLYSIS

Many neuro- and vasoactive peptides share proline residues in their sequence (**Table 4**). The presence of proline may not only determine the properties of the secondary structure necessary for their biological activity but also hinder non-specific proteolytic degradation (43). Alternatively, proline residues may serve as a recognition site for peptidases with restricted specificity for proline-containing substrates (**Table 5**).

The physiological importance of proline in peptide chains is obvious in the renin-angiotensin system, which regulates the arterial blood pressure. Here, angiotensin I is formed by the cleavage of angiotensinogen by renin (EC 3.4.23.15). Angiotensin I is cleaved by angiotensin-converting enzyme (ACE) (EC 3.4.15.1) with formation of the vasoactive angiotensin II. Renin is characterized by a restricted substrate specificity by cleaving exclusively a Leu-Leu bond in angiotensinogen, with an absolute requirement for the presence of a proline four residues prior to the cleavable site. The same proline prevents the degradation of angiotensin II after its formation from angiotensin I by ACE, preserving in this way its vasoactive capacity.

Substance P, bradykinin, and neurotensin are examples of the importance of proline for the proteolytic maturation of neuropeptides from their precursors or for the final degradation of these regulatory peptides. Proteolytic processing is trimmed by the presence of paired basic residues in the precursor molecule. A trypsin-like endopeptidase cleaves at these basic residues, eventually followed by the removal of the basic residue by a carboxy- or

TABLE 4. *Proline-containing neuro- and vasoactive peptides in human*

Angiotensin	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$
Vasopressin;	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH$_2$
Neurotensin	Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu
Neuropeptide Y (1–14)	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala
Peptide YY (1–14);	Tyr-Pro-Ile-Lys-Pro-Glu-Ala-Pro-Gly-Glu-Asp-Ala-Ser-Pro
Pancreatic polypeptide (1–14)	Ala-Pro-Leu-Glu-Pro-Val-Tyr-Pro-Gly-Asp-Asn-Ala-Thr-Pro
Luteinizing hormone-releasing hormone	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$
Thyrotropin-releasing hormone	pGlu-His-ProNH$_2$
Gastrin releasing peptide (1–10)	Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-
Corticotropin-releasing hormone (1–10)	Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-
Calcitonin (20–32)	His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-ProNH$_2$

TABLE 5. *Effects and functions of proline motifs*

MOTIF	EXAMPLES OF BIOLOGICAL FUNCTIONS
trans conformation of proline	
X-Pro-Y-	Protection against aspecific N-terminal degradation.
	Only recognized by aminopeptidase P (pre-Pro bond) or dipeptidyl peptidase IV (post-Pro bond).
X-Pro-Pro-Y-	Protection present in bradykinin, interleukin 6, factor XII, erythropoietin; possibly released by consecutive action of aminopeptidase P and dipeptidyl peptidase IV, or by prolyl oligopeptidase (post Pro-Pro bond)
X-Pro-Y-Pro-Z-	Processing to active metabolite of substance P, or to mature interleukin 6, by two step action of dipeptidyl peptidase IV, or by prolyl oligopeptidase (Pro-Z bond).
-X-Pro-Y	Motif recognized by carboxypeptidase P and prolylcarboxypeptidase.
-X-Pro-X-Y-Z	Blood pressure regulation: Pro prevents further degradation of angiotensin I by angiotensin converting enzyme.
-Pro-X-	Brain: hydrolysis of post-Pro peptide bond in hormones by prolyl oligopeptidase. Infections: destruction of hinge region of IgA by pathogenic bacteria (hydrolysis of post-Pro bonds).
-X-Pro-	HIV aspartic protease splits pre-Pro bonds in gag and gag-pol gene products to yield a series of proteins essential for viral replication. This motif is usually resistant to human protease.
-Gln-Arg-Pro	The preceding Gln makes the Arg-Pro bond susceptible to trypsin action.
-X-Pro-Pro-Y-	The post Pro-Pro bond is probably only sensitive to prolyl oligopeptidase in peptides consisting of less than 30 amino acids.
-X-Pro-Arg-Y-	Coagulation cascade: Pro mediates the action of thrombin on the post-Arg bond.
-Pro-Arg-Arg-Pro-	Neurotensin: prevention of aspecific hydrolysis of the dibasic sequence.
-Pro-Pro-/-Pro-Pro	The bond between two Pro residues, when not positioned at the N-terminus, possess a high degree of resistance to any human proteolytic enzyme.
cis-conformation of Proline	
-X-Pro-	Protein folding and intracellular signaling by peptidyl-prolyl *cis-trans* isomerases. Translocation of ions in transport channels.

aminopeptidase, which liberates the active peptide. In neurotensin, the dibasic sequence -Arg-Arg- is not cleaved because it is flanked by two proline residues. The precursor molecule of substance P is not cleaved behind the dibasic sequence in Arg-Arg-Pro because a proline residue follows.

In human plasma, substance P is rapidly converted to des (Arg1-Pro2)-substance P (fto des (Arg1-Pro2-Lys3-Pro4)-substance P (fsistent with the specificity of dipeptidyl peptidase IV (65). It has been demonstrated that rat nerve terminals and glial cells lack an active uptake system for substance P, but do possess a high-affinity transport system for the 5–11 fragment (66). The conversion of substance P to this metabolite by the action of dipeptidyl peptidase IV may therefore be of physiological importance. In agreement with a physiological role of dipeptidyl peptidase IV in substance P metabolism is the finding that tissues with the highest specific activities of dipeptidyl peptidase IV are largely those with a high substance P sensitivity or locations of substance-P synthesis.

In peptides or proteins with a penultimate Pro-Pro sequence, such as bradykinin (Table 4), IL-6, erythropoietin (Table 3), or coagulation factor XII (Ile-Pro-Pro-Trp-Glu-), the removal of the N-terminal amino acid is required before dipeptidyl peptidase IV can subsequently remove the Pro-Pro sequence, because this enzyme cannot cleave a Pro-Pro bond. Such a sequence may impose a combined action of aminopeptidase P and dipeptidyl peptidase IV for aminoterminal degradation (43, 67). The differences observed at the N-terminus of IL-6 seem to derive from the activities of aminopeptidase P and/or dipeptidyl peptidase IV (45).

On the other hand, as can be expected from its substrate specificities, prolyl oligopeptidase has the ability to cleave off the N-terminal X-Pro-Pro shield in a single step as demonstrated for bradykinin (68). The bond between proline residues, when not positioned at the amino-terminus, probably confers resistance to any human proteolytic enzyme.

In conclusion, the presence of proline in a peptide chain has, due to its unique cyclic and imino structure, an important influence on the conformation and folding of the peptide backbone. Functioning as a built-in signal, it contributes, by means of specific peptidases and *cis-trans* isomerases, to a diversity of regulatory, protective and destructive processes such as immunomodulation, coagulation, neuroendocrine homeostasis, inflammation, and microbial and viral infections. It is therefore not surprising that proline often tends to be highly conserved. FJ

The authors acknowledge the support of the Belgian Program on Interuniversity Poles of Attraction, the Flemish Biotechnology Program and the Belgian National Fund for Scientific Research.

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